THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 273, No. 41, Issue of October 9, pp. 26765–26771, 1998

Characterization of Two Human Genes Encoding Acyl Coenzyme A: Cholesterol Acyltransferase-related Enzymes*

(Received for publication, May 20, 1998, and in revised form, July 16, 1998)

Peter Oelkers‡§, Ajay Behari‡, Debra Cromley¶, Jeffrey T. Billheimer¶, and Stephen L. Sturley‡§‖

From the ‡Institute of Human Nutrition, §Departments of Pediatrics and Physiology and Molecular Biophysics, Columbia University College of Physicians and Surgeons, New York, New York 10032 and ¶DuPont Pharmaceutical Company, Experimental Station, Wilmington, Delaware 19880-0400

The enzyme acyl coenzyme A:cholesterol acyltransferase 1 (ACAT1) mediates sterol esterification, a crucial component of intracellular lipid homeostasis. Two enzymes catalyze this activity in *Saccharomyces cerevisiae* (yeast), and several lines of evidence suggest multigene families may also exist in mammals. Using the human ACAT1 sequence to screen data bases of expressed sequence tags, we identified two novel and distinct partial human cDNAs. Full-length cDNA clones for these ACAT related gene products (ARGP) 1 and 2 were isolated from a hepatocyte (HepG2) cDNA library. ARGP1 was expressed in numerous human adult tissues and tissue culture cell lines, whereas expression of ARGP2 was more restricted. *In vitro* microsomal assays in a yeast strain deleted for both esterification genes and completely deficient in sterol esterification indicated that ARGP2 esterified cholesterol while ARGP1 did not. In contrast to ACAT1 and similar to liver esterification, the activity of ARGP2 was relatively resistant to a histidine active site modifier. ARGP2 is therefore a tissue-specific sterol esterification enzyme which we thus designated ACAT2. We speculate that ARGP1 participates in the coenzyme A-dependent acylation of substrate(s) other than cholesterol. Consistent with this hypothesis, ARGP1, unlike any other member of this multigene family, possesses a predicted diacylglycerol binding motif suggesting that it may perform the last acylation in triglyceride biosynthesis.

The intracellular formation of sterol esters from fatty acid and sterol is mediated by acyl-CoA:cholesterol acyltransferase (ACAT).[1] The pathological accumulation of cholesterol esters in atherosclerotic lesions has lead to intense pursuit of ACAT inhibitors as pharmacological agents. Microsomal ACAT preparations from various tissues display differential sensitivities to some of these agents (1) including histidine modifiers (2). This suggests that more than one protein mediates the esterification reaction, such as occurs in yeast (reviewed in Ref. 3). *Saccharomyces cerevisiae* (budding yeast) has two ACAT related enzymes, Are1 and Are2, which are derived from separate genes and have been shown to independently esterify sterols (4, 5). In terms of contribution to the sterol ester mass of the cell, Are1 is the minor isoform relative to Are2. These genes were identified based on sequence conservation to a human gene, ACAT1, which encodes an ACAT enzyme with homologs in many mammalian species (6, 7). The human ACAT1 gene encodes a 550-amino acid polypeptide and is expressed in most tissues, predominantly placenta, lung, kidney, and pancreas (6). ACAT1 has been predicted to have two transmembrane domains (6) and has been immunolocalized to the endoplasmic reticulum (8, 9). When murine ACAT1 was disrupted in induced mutant mice, homozygotes for the deletion were found to essentially lack ACAT activity in embryonic fibroblasts and have negligible amounts of cholesterol ester in the adrenal cortex and peritoneal macrophages (10). However, cholesterol ester accumulation was normal in hepatocytes while dietary cholesterol absorption, an indirect marker for intestinal cholesterol esterification, was indistinguishable from control littermates. This is consistent with the concept of a multigene family for this activity.

ACAT isoenzymes may be required to perform the variety of physiological roles mediated by cholesterol esterification. Increases in cellular free cholesterol above certain levels are cytotoxic and are ameliorated by cholesterol ester formation (11). In hepatocytes, the bulk of cholesterol secreted in very low density lipoprotein is esterified intracellularly and determines apolipoprotein B secretion rates (12–14). Cholesterol esterification in the enterocyte may be necessary for cholesterol absorption from the lumen and secretion in chylomicrons into the lymph (15). The formation of cholesterol ester stores could also provide a readily available substrate for steroid hormone synthesis in steroidogenic tissues (16, 17). It is likely that different ACAT isozymes mediate each of these processes, and the data presented here support that hypothesis.

We reasoned that additional human ACAT proteins would have sequence similarity to regions conserved between human ACAT1 and yeast Are1 and Are2. (4). Accordingly, an ACAT consensus sequence was used to screen the data base of expressed sequence tags (dbEST). Several cDNA entries were identified which were transcribed from two independent human genes. This study is a description of the isolation of full-length cDNA clones for two ACAT-related gene products (ARGP1 and ARGP2), examination of their pattern of tissue

* This work was supported in part by a Grant-in-Aid/Investigatorship from the American Heart Association (NYC Affiliate) and by the Ara Parseghian Medical Research Foundation (to S. L. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF059202 and AF059203.

§ Supported by NHLBI, National Institutes of Health, Training Fellowship HL07343 in Arteriosclerosis.

‖ Established investigator of the American Heart Association. To whom correspondence should be addressed: Institute of Human Nutrition, Columbia University College of Physicians and Surgeons, 650 W. 168th St., New York, NY 10032. Tel.: 212-305-6304; Fax: 212-305-3079; E-mail: sls37@columbia.edu.

[1] The abbreviations used are: ACAT, acyl coenzyme A:cholesterol acyltransferase; ARGP, ACAT related gene product; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s); RT, reverse transcriptase; DEPC, diethylpyrocarbonate.

expression, and assays of enzymatic activity. We show that ARGP2 can catalyze the formation of sterol ester from cholesterol and oleoyl-CoA, leading us to rename this gene, ACAT2. By contrast, ARGP1 did not detectably esterify cholesterol and we propose that it performs acyl-CoA-dependent acylation f other molecules, such as diacylglycerol.

EXPERIMENTAL PROCEDURES

General—Molecular biology techniques were performed by conventional protocols (18, 19) and DNA modifying reagents were purchased from Life Technologies, Inc., New England Biolabs, or Promega as indicated. The Prime It random priming probe synthesis kit was obtained from Stratagene. The DIG Genius probe synthesis kit and CSPD were supplied by Boerhinger Mannheim. Radioactive reagents ([^{14}C]oleoyl-CoA and [^{32}P]dCTP) were purchased from NEN Life Science Products Inc. Ethidium bromide-stained agarose gels were visualized by the Kodak Digital Science 1D system. Automated DNA sequencing was performed at the Columbia University Cancer Center sequencing facility, and oligonucleotides were synthesized by Genset. DNA and amino acid sequence analysis and comparisons were performed using DNAStrider (20), PILEUP, and GAP programs (GCG Inc. (21)), Prosite (22), and Identify (Ref. 23, website http://dna.stanford.edu/identify/). Yeast media components were prepared as described (18).

Screening the dbEST—A 30-amino acid ACAT concensus peptide sequence (FAEMLRFGDRMFYKDWWNSTSYSNYYRTWN) was used as the query in a tblastn (which compares a protein sequence against a nucleotide sequence data base translated in all reading frames (24, 25)) search of the data base of expressed sequence tags at NCBLI (dbEST). Three clones, H24971, R07932, and R99213, derived from a common gene (named ACAT related gene product 1, ARGP1), were identified ($p < 10^{-4}$). The entire human ACAT1 protein was then used in an identical search. In addition to clones of ACAT1 and ARGP1, two entries, R10272 and W76421, with significant similarity were identified ($p < 10^{-5}$). They were derived from a gene we named ARGP2. Rescreening the dbEST with these clones identified two more ARGP2 entries. *Escherichia coli* clones with the largest inserts corresponding to these sequences were obtained from the I.M.A.G.E. consortium and resequenced with T3, T7, or gene specific primers.

5′ Rapid Amplification of cDNA Ends (RACE) of ARGP1—Oligo(dT) primed, double stranded cDNA was reverse transcribed from human, ileal, poly(A)$^+$ mRNA, kindly provided by Dr. Paul Dawson, and ligated to adapters using a commercially available kit (CLONTECH, Palo Alto, CA). Touchdown PCR (26) was performed for 35 cycles with a forward primer complementary to the adapter (AP1, 5′-CCATCCTAATACGACTCACTATAGGGC) and a reverse primer (End4A, 5′-CCACCTGGAGCTGGGTGAAGAAC) complementary to the ARGP1 dbEST clone Z43867. The PCR mixture included 200 nM each oligo, 200 μM dNTPs, 1.75 mM $MgCl_2$, 2.5 units of *Taq*, and the cDNA diluted 1:500. The 700-bp reaction product was gel isolated, ligated into YEp352 with a T overhang generated by *Taq* polymerase, and sequenced.

5′ RACE of ARGP2—A human, fetal (20 weeks post-conception) liver/spleen, oligo(dT)-primed, cDNA library in the vector pT7T3D was kindly provided by Dr. Bento Soares. PCR was performed with this cDNA, a forward primer (M13 reverse, 5′-TGAGCGGATAACAATTTCACACAGG) complementary to the vector and a reverse primer (20S, 5′-CCCCATGCTGAGGTCTGTGATCAG), complementary to the ARGP2 dbEST clone R10272, using the above conditions. The 800-bp reaction product was gel isolated, ligated into pBS:SK (Stratagene) with a T overhang generated by *Taq* polymerase, and sequenced.

Hybridization Screening of a HepG2 cDNA Library—A yeast expression library of HepG2 cDNA (size selected for inserts greater than 2.5 kb in pAB23BXN, commercially available from Austral Biologicals, San Ramon, CA), was propagated in the *E. coli* strain MC1061 and plated onto 135-mm LB + ampicillin (50 μg/ml) plates at an approximate density of 5000 colonies per plate. Membrane (Hybond-N, Amersham) replicas of the plates were probed by hybridization with a digoxigenin-labeled probe specific for ARGP1 (synthesized using a 420-bp *Not*I, *Pst*I digestion product of the 5′ RACE product) or ARGP2 (synthesized using the 5′ RACE product) in 5 × SSC, 0.05% SDS, 0.1% *N*-laurolysarcosine, 0.1 mg/ml salmon sperm DNA, and 2% (w/v) blocking reagent (Boerhinger Mannheim) at 65 °C for 14–18 h. The membranes were washed in 0.2 × SSC, 0.1% SDS at 60 °C for 80 min, incubated with an anti-digoxigenin antibody (1:10,000), washed in Tris-buffered saline, incubated with the peroxidase substrate CSPD (Boerhinger Mannheim), and detected by enhanced chemiluminescence (ECL). For ARGP1, 8 single positive clones were isolated after screening ~20,000 clones. For ARGP2, 4 single positive clones were isolated after screening ~30,000 clones. The longest clones for each were sequenced multiple times on both strands using vector and gene-specific oligonucleotides.

Tissue Culture—Cultured human Caco2, HeLa, HepG2, and THP1 cell lines were donated by Dr. R. J. Deckelbaum and originally obtained from the ATCC. HepG2, HeLa, and Caco2 cells were maintained as cell monolayers in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) + 10% fetal bovine serum (HyClone) in 5% CO_2. THP1 monocyte cells were maintained in suspension in RPMI (Life Technologies, Inc.) + 10% fetal bovine serum in 5% CO_2. Differentiation of THP1 cells was stimulated with 150 ng/ml tetramyristate phorbol ester and 140 μM β-mercaptoethanol. Whole cell RNA was isolated from confluent monolayer cultures or pelleted THP1 cells using TRIzol (Life Technologies, Inc.) extraction. The Caco2 cells had been confluent for approximately 21 days.

Human Adult and Fetal Multi-tissue Northern Blot Analysis—Commercially obtained multi-tissue Northern blot (CLONTECH) contained 2 μg of poly(A)$^+$ RNA from human adult or fetal (18–24 weeks postconception) tissues originally resolved on a 1.2% agarose, formaldehyde gel. The adult tissue membrane was hybridized with a random-hexamer primed, [^{32}P]dCTP-labeled probe, generated using the insert of the ARGP1 dbEST clone R99213, in ExpressHyb buffer (CLONTECH) for 1 h at 68 °C. The membrane was washed in 0.1 × SSC, 0.5% SDS at 50 °C. After stripping the membrane was probed with ARGP2 (dbEST clone 10272 insert and the ARGP2 5′ RACE product) using the conditions above. The fetal tissue Northern blot was hybridized with the same ARGP2 probe.

Reverse Transcription PCR—Human cDNA obtained as part of a Quick Screen cDNA Panel of Human tissues (CLONTECH) or reverse transcribed (Life Technologies, Inc. kit) from human ileal poly(A)$^+$ mRNA was used as the template in a PCR reaction with primers specific for ARGP1 (106, GGCATCCTGAACTGGTGTGTGGTG; 110, AGCTGGCATCAGACTGTGTCTGG), ARGP2 (202, GAGTTCCCCCACATTCATCAAATCC; 206, CATGCTGCTGCTCATCTTCTTTGCA), or β-actin (Act1, GAGCTGCCTGACGGCCAGGTC; Act2, CACATCTGCTGGAAGGTGGACAG). The PCR mixture included 1.5 mM $MgCl_2$, 200 μM dNTPs, 400 nM of each primer, and 2 units of *Taq* (Life Technologies, Inc.). Following 35 cycles (94 °C, 45 s; 60 °C, 45 s; 72 °C, 2 min), the products were resolved on a 1% agarose, 0.5 μg/ml ethidium bromide gel. For RNA prepared from human cultured cells, first strand cDNA synthesis was performed, with and without reverse transcriptase (SuperscriptII, Life Technologies, Inc.) using 4 μg of whole cell RNA. A fraction of each reaction (10%) was the template in a PCR reaction (30 cycles of 94 °C, 30 s; 68 °C, 2 min) with primers specific for ARGP1 (103, GCTTCATGGAGTTCTGGATGGTGG; 106, GGCATCCTGAACTGGTGTGTGGTG), ARGP2 (201, GACACCTCGATCTTGGTCCTGCC; 202, as above) or human ACAT1 (ACATa, CGGAATATCAAACAGGAGCCCTTC; ACATb, CATTCCAAAGAACATGAAGATGCACG).

In Vitro Assay of ACAT Activity in Yeast Microsomes—The cDNA inserts of the longest ARGP1 and ARGP2 HepG2 library clones were removed by *Not*I, *Eco*RI digestion and ligated into the yeast expression vector pRS426GP which utilizes the galactose inducible GAL1/GAL10 promoter. A cDNA corresponding to the coding region of human ACAT1 flanked by 5 bp of 5′-untranslated region and 1 bp of 3′-untranslated region, in pRS426GP was described previously (27). Yeast strain, SCY059 (MAT*a*, *ade2-1, can1-1, trp1-1, ura3-1, his3-11, 15, leu2-3, 112, met14Δ14HpaI-SalI, are1ΔNA::HIS3, are2Δ::LEU2*) with deletions in *ARE1* and *ARE2*, the yeast homologs of human ACAT1 (4), was transformed with the above constructs or pRS426GP using lithium acetate and nucleic acid prototrophy selection (28). Expression of the constructs was verified by RT-PCR analysis of RNA isolated from the transformed cells. Culturing of the transformed yeast, induction of expression, microsome isolation, and sterol esterification assays were as described previously (27). In those experiments involving diethylpyrocarbonate (DEPC) to modify histidine residues, a preincubation with 100 μM DEPC was performed as described (2).

RESULTS

Isolation of Full-length cDNA Clones for Two ACAT Related Human Genes—A comparison of the human ACAT1 protein and the two yeast ACAT orthologs (Are1, Are2) identified a highly conserved (70% identical) region of 30 amino acids (ACAT1 amino acids 391–420) near the carboxyl terminus. This peptide was used to screen the data base of expressed sequence tags (dbEST). The search identified several human cDNAs, the longest being 890 bp (GenBank accession number H45923), derived from a common gene we call the ARGP1. To

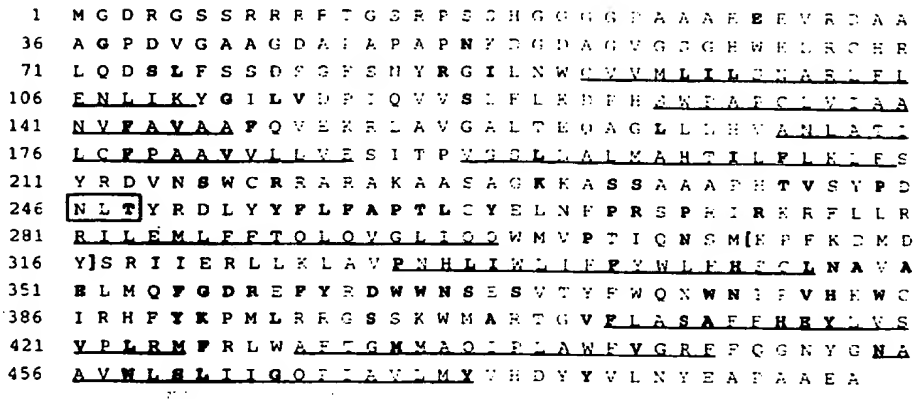

FIG. 1. **ARGP1 predicted peptide sequence.** A 1976-bp ARGP1 cDNA clone was identified by colony hybridization screening of a HepG2 cDNA library. Translation of this clone predicts the 488-amino acid peptide shown. The residues in *bold* are conserved with human ACAT1. The *underlined* portions are predicted transmembrane domains, a potential *N*-linked glycosylation site is *boxed*, and a putative tyrosine phosphorylation motif is in *brackets*. The sequence has been deposited at GenBank, accession number AF059202.

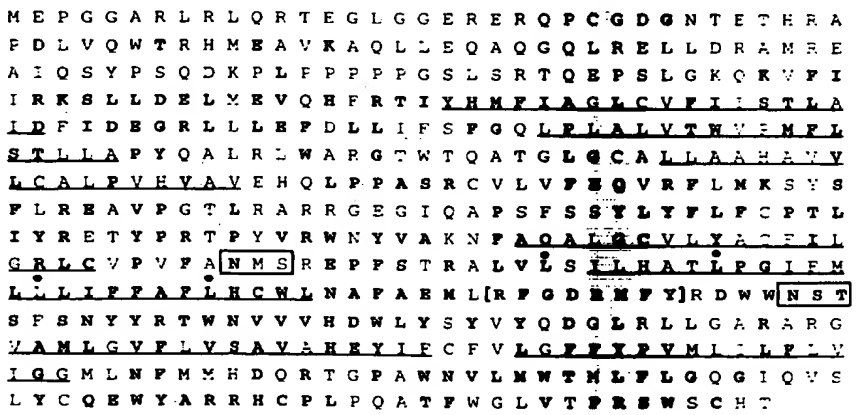

FIG. 2. **ARGP2 predicted peptide sequence.** A 2040-bp ARGP2 cDNA isolated by screening a HepG2 cDNA predicts the 522-amino acid polypeptide shown. The residues in *bold* are conserved with human ACAT1. The *underlined* portions are predicted transmembrane domains, two potential *N*-linked glycosylation sites are *boxed*, a putative tyrosine phosphorylation motif is in *brackets*, and the *circles* mark the leucine zipper heptad motif. The sequence has been deposited at GenBank, accession number AF059203.

date, 26 clones for human ARGP1 are present in the dbEST from fetal liver/spleen, infant brain, breast, cerebellum, hippocampus, kidney, placenta, testis, ovary tumor, colon tumor, and lung tumor libraries, suggesting ubiquitous and abundant expression. In addition, ARGP1 is also represented as several murine entries (*e.g.* GenBank accession number C75990). The dbEST was then searched using the entire ACAT1 protein sequence. Four human cDNAs, distinct from ARGP1 cDNA clones, were identified in fetal liver/spleen and fetal heart libraries and are derived from a common gene we call ARGP2. The longest entry was 600 bp (GenBank accession number R10272). To date these are the only dbEST entries for human ARGP2, although several murine entries have been identified (*e.g.* GenBank accession number AA410072).

Northern blot analysis of human tissues (Figs. 3 and 4) showed that the initial dbEST clones for ARGP1 and ARGP2 were truncated, relative to the observed transcripts, by approximately 1000 and 1400 bp, respectively. To isolate full-length cDNA clones, 5′ RACE was performed using cDNA synthesized from human liver (ARGP1) or ileal (ARGP2) mRNA but yielded only 600 nucleotides of novel sequence for each. The respective 5′ RACE products were then used as probes to screen a size selected (>2.0 kb), HepG2 cDNA library by hybridization. The longest ARGP1 clone contained 1976 nucleotides and a 19-base poly(A)$^+$ tract which agreed with the length of the minimal ARGP1 transcript detected by Northern blot (Fig. 3). HepG2 cells express only the 2.0-kb ARGP1 transcript.[2] A similar approach identified ARGP2 clones, the longest of which contained 2040 bp of sequence with a 50-base poly(A)$^+$ tract in accordance with the observed length of the ARGP2 transcript (Fig. 4).

ARGP1 Predicted Peptide—The longest open reading frame of ARGP1, flanked by a 244 nucleotide 5′-untranslated region and a 265-nucleotide 3′-untranslated region, encodes a 488-amino acid protein (Fig. 1) with a calculated molecular mass of 55,216 daltons. The predicted initiator methionine lies within a consensus for initiation of translation (29) and downstream of an in-frame termination codon. Comparison to ACAT1 revealed 22% amino acid sequence identity (29% similarity) over the entire molecule. The conservation of these molecules is greatest toward the COOH terminus, such that ACAT1 and ARGP1 are 28% identical over the last 250 residues. This pattern of sequence similarity is strikingly similar to that observed from comparison of ACAT1 with the yeast Are1 and Are2 proteins. ARGP1 is predicted to be a membrane bound protein with nine putative transmembrane domains and one *N*-linked glycosylation site. Uniquely, ARGP1 contains a diacylglycerol/phorbol ester binding signature sequence (H.[FWY]..[KR].F..P) at amino acids 382–392 which was originally identified by comparison of protein kinase C isoforms and diacylglycerol kinases (Fig. 7) (36, 37)). This motif is also conserved in the murine homolog of ARGP1 residing at the dbEST (GenBank accession number AA764382).

ARGP2 Predicted Peptide—The longest ARGP2 open reading frame, flanked by a 51-nucleotide 5′-untranslated region and a 420-nucleotide 3′-untranslated region, predicts a 522-amino acid protein with a calculated molecular mass of 59,942 daltons (Fig. 2). The predicted initiator methionine lies within a consensus for initiation of translation (29). Over the entire molecule, the predicted protein is 47% identical (54% similar) to human ACAT1. This conservation is even more pronounced at the COOH-terminal end of the molecules, raising to 63% identity over the last 250 residues. ARGP2 is predicted to be a membrane bound protein with seven putative transmembrane domains and two *N*-linked glycosylation sites. ARGP2 is similar to ACAT1 in that it contains a leucine zipper (338–359) which may mediate multimerization or interaction with other proteins. ARGP2 does not pos-

[2] T. Seo, P. Oelkers, M. Giattina, R. J. Deckelbaum, and S. L. Sturley, manuscript in preparation.

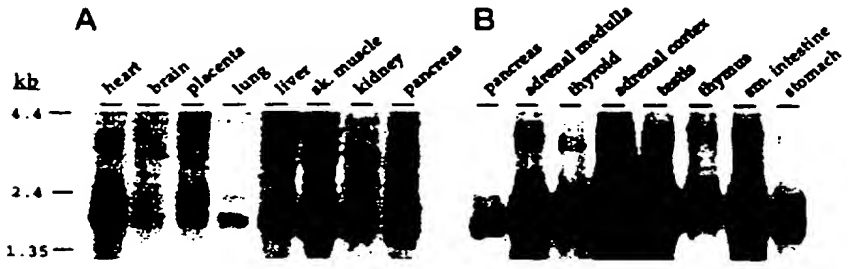

FIG. 3. **Northern blot analysis of ARGP1 expression in human adult tissues.** 2 μg of mRNA from human adult tissues (*panel A*, CLONTECH MTN I; and *panel B*, endocrine system MTN) was hybridized with a [^{32}P]dCTP, random-hexamer labeled, human ARGP1 probe in Express Hyb solution for 1 h at 68 °C. After washing in 0.2 × SSC, 0.1% SDS at 60 °C for 40 min, the membranes were exposed to x-ray film. Molecular weight markers were as supplied by CLONTECH.

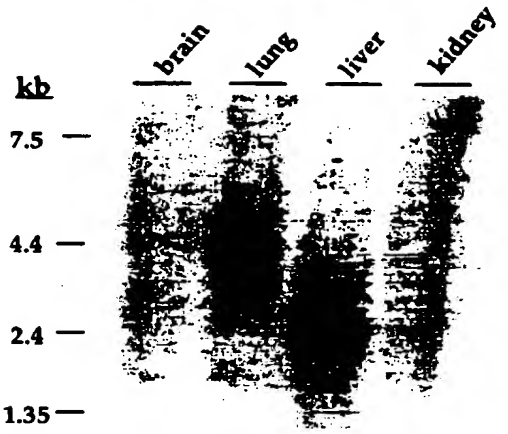

FIG. 4. **Northern blot analysis of ARGP2 expression in human fetal tissues.** 2 μg of mRNA from human fetal tissues (CLONTECH Fetal MTN II) was resolved on a denaturing, 1.2% agarose gel, transferred to a nylon membrane, and hybridized with a [^{32}P]dCTP, random-hexamer labeled, human ARGP2 probe in Express Hyb solution for 1 h at 65 °C. After washing in 0.2 × SSC, 0.1% SDS at 68 °C for 40 min, the membranes were exposed to x-ray film. Molecular weight markers were as supplied by CLONTECH.

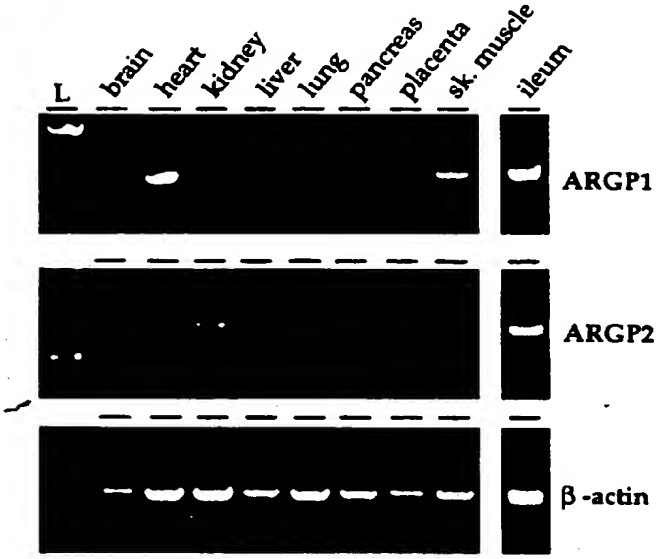

FIG. 5. **Analysis of ARGP1 and ARGP2 expression in adult human tissues using RT-PCR.** PCR was performed as described using a Quick Screen Human cDNA Panel (CLONTECH), or cDNA reverse transcribed from human ileal poly(A)$^+$ mRNA, and primers specific for ARGP1, ARGP2, or β-actin in a standard PCR mixture. The PCR products, predicted to be 921 (ARGP1), 844 (ARGP2), or 835 (β-actin) bp, were resolved on ethidium bromide-stained agarose gels with a 100-bp DNA ladder (*L*; Life Technologies, Inc.).

sess a predicted diacylglycerol/phorbol ester-binding site. A sequenced tag entry (number WI-11660) for ARGP2 localizes to human chromosome 12, further distinguishing it from ACAT1, which is located on chromosome 1 (30).

ARGP1 and ARGP2 Expression in Human Tissues and Tissue Culture Cell Lines—Expression of a second ACAT would be expected in tissues (*e.g.* liver and intestine) which exhibit normal ACAT activity in the induced mutant ACAT1 (*acact*$^-$) mice (10). Expression of ARGP1 and ARGP2 was thus examined by Northern blot of human RNA (Figs. 3 and 4). Hybridization of an ARGP1 cDNA probe to a panel of adult tissue mRNAs detected a transcript in all tissues examined (Fig. 3). However, ARGP1 expression levels varied qualitatively among tissues with moderate expression in thyroid, stomach, heart, skeletal muscle, and liver and high expression in adrenal cortex, adrenal medulla, testes, and small intestine. The presence of a 2.0-kb transcript was invariable among the tissues while a 2.4-kb transcript was observed in about half the tissues, most notably the tissues with high expression. The origin of these two transcripts has not been identified, however, their heterogeneity is unlikely to lie at the 3′ end of the message since all dbEST entries for ARGP1 cDNAs terminate at a similar position. Hybridization of the same membrane, under identical conditions, with an ARGP2 cDNA probe failed to detect a transcript in any tissue (data not shown). Since th four ARGP2 dbEST clones were in human fetal libraries, ARGP2 expression was examined in human fetal tissues by Northern blot (Fig. 4). A 2.2-kb transcript was detected in fetal liv r but not in fetal brain, lung, or kidney.

To further examine the expression of ACAT2 in adults, a RT-PCR was performed using cDNA generated from a variety of tissues (Fig. 5). As shown, ARGP2 is expressed in human adult heart, kidney, liver, lung, pancreas, and ileum. The identity of the PCR product was verified by Southern blotting and hybridization with an ARGP2-specific cDNA probe (data not shown). An RT-PCR analysis of ARGP1 on these same samples gave a similar pattern of expression to that determined by the Northern blot in Fig. 3.

ARGP1 and ARGP2 expression in human tissue culture cell lines was also examined by RT-PCR (Fig. 6). ARGP1 was expressed in cell culture models for human endothelial (HeLa), hepatocyte (HepG2), monocyte (undifferentiated THP1), macrophage (differentiated THP1), and intestinal epithelial (Caco2) cells. Expression of ARGP2 was limited to HepG2 and Caco2 cells. This reinforces the concept that ARGP1 is widely expressed while the expression of ARGP2 is more restricted. ACAT1 was expressed in all of these cell lines confirming previous observations (7, 31) (data not shown).

Assay of ACAT Activity in ACAT Negative Yeast Transformed with ARGP1 and ARGP2—The ability of ARGP1 and ARGP2 to esterify sterols was assayed in a sterol esterification deficient yeast strain (SCY059) in which the endogenous *ARE* genes were deleted (27). Microsomes from these yeast, transformed with an expression vector harboring no insert or cDNA inserts for ARGP1, ARGP2, or human ACAT1 were assayed *in vitro* for the incorporation of [^{14}C]oleate into sterol ester. Since we previously demonstrated that cholesterol is the preferred substrate for mammalian ACAT enzymes (27, 32), assays were performed with exogenous cholesterol supplied in Triton WR-1339. As shown in Table I, ARGP2 forms cholesterol ester at a

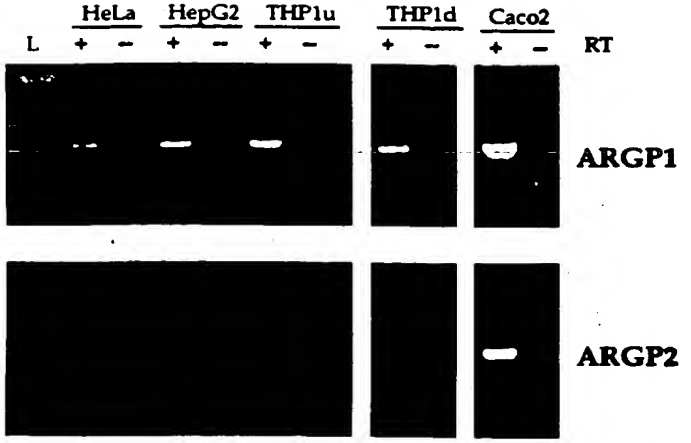

FIG. 6. **Analysis of ARGP1 and ARGP2 expression in tissue culture cells using RT-PCR.** Monolayer cultures of HeLa, HepG2, undifferentiated THP1, and Caco2 tissue culture cells were grown as described under "Experimental Pprocedures." THP1 cells were differentiated into macrophages by the addition of phorbol ester. Total cellular RNA was isolated from the cells and reverse transcribed using oligo(dT) priming in parallel with reactions which lacked RT enzyme. Oligonucleotide pairs complementary to ARGP1 or ARGP2 were included in a PCR using the conditions described in the legend to Fig. 5. The PCR products, predicted to be 667 (ARGP1) and 352 (ARGP2) bp, were resolved on an ethidium bromide-stained agarose gel alongside a 100-bp DNA ladder (*L*; Life Technologies, Inc.).

TABLE I

In vitro analysis of ACAT activity of transformed yeast

S. cerevisiae strain SCY059 (*are1*$^-$*are2*$^-$) was transformed with the yeast expression vector pRS426GP harboring either no insert or cDNAs encoding human ACAT1, ARGP1, or ARGP2. Expression was under the control of the inducible *GAL1/10* promoter. Microsomes were isolated from galactose induced yeast cultures and incubated in 0.1 M sodium phosphate, 1 mM glutathione, 20 nM [^{14}C]oleate, with exogenous cholesterol (260 μM in Triton WR-1339) for 3 minutes at 37 °C. The amount of radioactivity incorporated into sterol ester was determined by thin layer chromatography and scintillation counting as described. In those experiments involving DEPC, microsomes were preincubated at room temperature in the presence or absence of 100 μM DEPC for 30 min prior to the ACAT assay. Data are pmol cholesteryl oleate formed per min/mg of protein expressed as mean ± S.E. from at least three different experiments on different preparations or of a representative experiment performed in triplicate.

cDNA Expressed	In vitro microsomal ACAT activity[a]	Active site modification[b]		
		Minus DEPC	Plus DEPC (100 μM)	Degree of inhibition
No insert	2 ± 2			
ACAT1	340 ± 76	340 ± 16	69 ± 4	80%
ARGP1	4 ± 3			
ACAT2	49 ± 15	45 ± 5	30 ± 1	33%

[a] Representative of three different experiments on different preparations.

[b] Representative experiment performed in triplicate.

rate of 49 pmol/min/mg of microsomal protein. This is 24-fold over background and about 15% of the activity detected in microsomes from ACAT1 transformants. We therefore renamed ARGP2 as ACAT2. ARGP1 did not display significant ACAT activity. None of the enzymes showed the ability to use ergosterol, the major sterol in yeast microsomes, as a substrate (data not shown). While the ACAT1 and ACAT2 mediated activities were equally sensitive (75% inhibition) to th ACAT inhibitor Dup128 (0.5 μM; not shown), they showed significantly different sensitivity to the histidine/tyrosine modifying agent diethylpyrocarbonate (DEPC, Table I). This reagent was previously demonstrated to distinguish liver and adrenal ACAT activities, the latter being significantly more sensitive. Since adrenal ACAT would primarily represent ACAT1, our data are consistent with ACAT2 representing the DEPC-resistant isoform identified by Kinnunen *et al.* (2).

DISCUSSION

We have isolated two independent human cDNAs, ARGP1 and ACAT2, which encode proteins with significant sequence similarities to human ACAT1. The level of nucleotide sequence conservation between ACAT1 and ACAT2 (55%) suggests their common evolution possibly arising from a gene duplication event, as clearly occurred in the case of the yeast *ARE* gene family. However, ARGP1 is more distantly related, bearing 39 and 43% nucleotide identity with ACAT1 and ACAT2, respectively, and may have evolved independently. The uniform similarity between the human genes and the two yeast *ARE* genes precludes any assignment of lineage across species.

The similarity among the three human ACAT-like proteins is most distinct over their COOH-terminal regions just as is the case when comparing the yeast Are proteins to ACAT1. The predicted ARGP1 protein displays 28% identity with ACAT1 over this portion of the molecule and includes a FY.DWWN motif present in all cloned ACATs and shown to be important for enzymatic activity (Fig. 7A).[3] However, ARGP1 is the most divergent member of this gene family. For example, a HSF motif (residues 268–270) is invariant in ACAT1 and yeast Are enzymes and was critical to ACAT1 activity in CHO cells. Replacement of Ser by Leu produced an inactive and unstable molecule (33). This motif is not conserved in ARGP1, although several serines are present in the region (*e.g.* Ser^{227}, Fig. 7*B*). ARGP1 is also unique in its predicted possession of a diacylglycerol/phorbol ester-binding site (Fig. 7*A*), leading us to speculate that this enzyme might esterify diacylglycerol to produce triglyceride. Sequence similarity between diacylglycerol acyltransferase and ACAT enzymes might be expected since both have a common substrate, acyl-CoA, but differ in the alcohol (cholesterol or diacylglycerol) used as a second substrate.

Of the two new gene products described here, ACAT2 displays significantly greater sequence similarity to ACAT1, with an overall identity of 47% and 63% invariance over the COOH-terminal half of the molecules. The FY.DWWN motif common to this family of proteins is maintained in ACAT2 to the extent that the flanking residues render the tyrosine a candidate for phosphorylation as observed in ACAT1 and in yeast (Fig. 7*A*). Tyrosine phosphorylation may be a regulator of ACAT activity, although serine and threonine phosphorylation is unlikely to be involved (34, 35). The HSF motif found in ACAT1, Are1 and Are2 is conservatively replaced in ACAT2 by YSF (residues 244–246; Fig. 7*B*). Interestingly, histidine modifying agents selectively inactivate adrenal microsomal ACAT activity but display a significantly higher K_i (1500 *versus* 250 μM) against liver microsomes (2). It is intriguing to speculate that sequence variation in the (H/Y)SF motif may explain this observation. In accordance with this, we showed that ACAT1 was significantly more sensitive to DEPC than ACAT2. In common with ACAT1, Are1 and Are2, the ACAT2 sequence predicts a leucine heptad motif which may play a role in multipeptide complex formation. Radiation inactivation studies in rat liver microsomes have shown that the ACAT enzymatic complex is about 200 kDa (36, 37), much larger than the predicted monomer for ACAT 1 (65 kDa) or ACAT2 (60 kDa). There is also evidence that ACAT1 interacts with itself in a yeast two-hybrid system (38) and ACAT2 may be similar in this regard. ARGP1 and ACAT2 are also similar to ACAT1 in terms of hydrophobicity. While previous studies suggested that ACAT1 contains two transmembrane domains (6), the PredictProtein algorithm (39) indicates

[3] Z. Guo, D. Cromley, J. T. Billheimer, and S. L. Sturley, manuscript in preparation.

A The "DWWN" region of the ACAT g ne family

```
ARE1   482   ELtRFaDRyF YgDWWNcvSf eeFsRiWNVP VHKfLlRHVY hssmgal.hl sKS
ARE2   514   ELtRFGDRyF YgDWWNcvSw adFsRiWNiP VHKfLlRHVY hssmssf.kl nKS
ACAT1  394   EmlRFGDRmF YkDWWNStSy snyyRtWNVv VHdWLyyyaY kdfLwffskr fKS
ACAT2  368   EmlRFGDRmF YrDWWNStSf snyyRtWNVv VHdWLysyVY qdgLrllgar arg
ARGP1  351   ELmqFGDReF YrDWWNSeSv tyFwqnWNiP VHKWciRHfY kpmLrrgss. .Kw
                         π                      *   *
Consensus    EL-RFGDR-F Y-DWWNS-S- --F-R-WNVP VHKWL-RHVY ---L------ -KS
```

B The "HSF" region of the ACAT gene family

```
ARE1   306   FvMKSHSFAf yNgyLWdIkq
ARE2   335   lLMKmHSFAf yNgyLWgIke
ACAT1  265   FvMKaHSFvr eNvprvLnsA
ACAT2  240   FLMKSySFlr eavpgtL.rA
ARGP1  206   FL.KlfSyrd vNswcr..rA
                    σ
Consensus    FLMKSHSFA- -N--LWLI-A
```

FIG. 7. **Consensus sequences in the ACAT multigene family. Two regions** of structural and functional conservation are shown. The amino acid position of each initial residue is shown. *Uppercase* residues **indicate those** of the consensus calculated with a plurality of 2. *A*, The DWWN region. The FY.DWWN motif is invariant in all members **identified to date** of this gene family, the tyrosine and tryptophans being critical to activity.[3] In all but ARGP1, the Tyr constitutes a candidate **target for phosphorylation** (indicated in *bold* and by π). In ARGP1, the *underlined* sequence HKWCIRHFYKP represents a candidate for diacylglycerol **binding** as found in protein kinase C and diacylglycerol kinases (motif, H.[FWY]..[KR].F..P). The *asterisks* identify those residues critical to **definition** of this motif that distinguish ARGP1 from the other members of the family. *B*, the HSF region. The central serine residue (indicated σ) **was found** to be critical to the activity and stability of Chinese hamster ovary ACAT1.

eight such domains in ACAT1, similar to the number predicted for ARGP1 (nine) and ACAT2 (seven). Membrane spanning domains are expected characteristics of ACAT and diacylglycerol acyltransferase enzymes since both activities are associated with microsomal membranes (40–42).

In addition to sequence similarity with ACAT1, we expect alternate ACAT enzymes to be expressed in the tissues which retain ACAT activity in the induced mutant ACAT1 mouse, namely the liver and intestine. ARGP1 met this criteria, however, it is also highly expressed in human adult adrenal cortex which was depleted of cholesterol esters in the induced mutant mouse. Monocytes from *acact*$^-$ mice were also devoid of cholesterol ester and yet ARGP1 mRNA was detected in the human THP1 monocyte cell line. This evidence is contrary to ARGP1 being an ACAT, barring species-specific differences in expression. By the sensitive technique of RT-PCR, ACAT2 expression was observed in human adult liver and intestine and in cell culture models of the hepatocyte and intestinal enterocyte but was undetectable in THP1 monocytes and macrophages. This profile of expression is consistent with a role for ACAT2 in the livers and intestine of mammals, particularly ACAT1 knockout mice.

In confirmation of ACAT2 being a candidate for a second ACAT, heterologous expression of ACAT2 in an ACAT-negative yeast strain conferred significant microsomal cholesterol esterification with oleoyl-CoA at a level comparable to the 20–50 pmol/min/mg of protein observed in human liver microsomes supplied with exogenous cholesterol (43). The ACAT2-mediated esterification activity was significantly (85%) less than that mediated by ACAT1 in yeast. This may be due to differences in protein expression (although both mRNAs were produced at high levels as detected by RT-PCR, data not shown), protein stability, or a genuine difference between the two enzymes.

Liver ACAT, predicted to comprise both ACAT1 and ACAT2, utilizes a limited range of sterol substrates but a wid variety (16:0, 18:0, 18:1, 18:2, and 20:4) of fatty acyl-CoAs (27, 44). Determining substrate-specific differences between ACAT1 and ACAT2 may thus explain their redundancy. Th redundancy may also be related to substrate affinity such as seen between the hexokinase types I-III and hexokinase type IV (glucokinase) (45). In such a scenario, one ACAT would have a lower affinity for cholesterol and only catalyze esterification at high cholesterol concentrations.

In addition to potential differences in activity, the two enzymes may have different physiological roles. For storage, cholesterol esters concentrate as cytoplasmic neutral lipid droplets, whereas for lipoprotein synthesis, cholesterol esters are incorporated into lipoprotein particles in the endoplasmic reticulum lumen. Redundant ACAT enzymes might allow one to be specific for cytoplasmic release of the cholesterol ester product and another to mediate endoplasmic reticulum lumenal release. Since lipoprotein synthesis occurs primarily in the liver and intestine, we speculate that ACAT2 may release cholesterol ester into the endoplasmic reticulum lumen, leaving ACAT1 to esterify and store sterols in the cytoplasm. The large amount of cholesterol ester, likely as cytoplasmic droplets, in the livers of high fat, high cholesterol fed *acact*$^-$ mice, is contrary to this hypothesis. Alternatively, ACAT2's role may be important in the fetus since it was easily detected by Northern blot in human fetal liver.

The abundance of ARGP1 entries in the dbEST from a wide variety of cDNA libraries is reflective of the ubiquitous nature of ARGP1 expression in human adult tissues and tissue culture cell lines. This suggests that ARGP1 serves a function important to many cell types. Expression of two independent clones of ARGP1 under the regulation of two yeast promoters, *GAL1/10* and *GAPDH* (not shown), failed to detectably esterify cholesterol or ergosterol. ARGP1-specific mRNA was identified by RT-PCR in each case. We take this as further evidence that unlike ACAT1 and ACAT2, ARGP1 is not involved in cholesterol esterification, at least when expressed in yeast. Based on the conservation of amino acids in ARGP1 that are important for ACAT1 to be active, ARGP1 likely catalyzes a reaction similar to ACAT. Other esterification reactions which use fatty-acyl CoAs as substrates include retinol esterification, methyl ester formation, triterpene esterification, monoacylglycerol transferase, and diacylglycerol transferase. In the latter case our observations of a diacylglycerol-binding site in ARGP1 biases us to the possibility of ARGP1 being diacylglycerol acyltransferase, which to date has not been isolated at the molec-

ular level. We are presently investigating whether ARGP1 can mediate these reactions.

Acknowledgments—We are grateful to Dr. B. Soares for helpful advice, Fanny Keyserman for technical assistance, and Dr. J. J. Rich for advice regarding this manuscript.

REFERENCES

1. Maduskie, T., Billheimer, J., Germain, S., Gillies, P., Higley, C., Johnson, A., Pennev, P., Shimshick, E., and Wexler, R. (1995) *J. Med. Chem.* **38,** 1067–1083
2. Kinnunen, P. M., DeMichele, A., and Lange, L. G. (1988) *Biochemistry* **27,** 7344–50
3. Sturley, S. L. (1997) *Curr. Opin. Lipidol.* **8,** 167–173
4. Yang, H., Bard, M., Bruner, D. A., Gleeson, A., Deckelbaum, R. J., Aljinovic, G., Pohl, T., Rothstein, R., and Sturley, S. L. (1996) *Science* **272,** 1353–1356
5. Yu, C., Kennedy, N. J., Chang, C. C. Y., and Rothblatt, J. A. (1996) *J. Biol. Chem.* **271,** 24157–24163
6. Chang, C. C. Y., Huh, H. Y., Cadigan, K. M., and Chang, T. Y. (1993) *J. Biol. Chem.* **268,** 20747–20755
7. Uelman, P. J., Oka, K., Sullivan, M., Chang, C. C. Y., Chang, T. Y., and Chan, L. (1995) *J. Biol. Chem.* **270,** 26192–26201
8. Chang, C. C., Chen, J., Thomas, M. A., Cheng, D., Del Priore, V. A., Newton, R. S., Pape, M. E., and Chang, T.-Y. (1995) *J. Biol. Chem.* **270,** 29532–29540
9. Tabas, I., Zha, X., Maxfield, F., Chang, T.-Y., Beatini, N., Farese, R. V., and Meiner, V. (1996) *Circulation* **94,** I-35
10. Meiner, V. M., Cases, S., Myers, H., Sande, E. R., Bellosta, S., Schambelan, M., Pitas, R. E., McGuire, J., Herz, J., and Farese, R. V. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 14041–14046
11. Warner, G. J., Stoudt, G., Bamberger, M., Johnson, W. J., and Rothblatt, G. H. (1995) *J. Biol. Chem.* **270,** 5772–5778
12. Carr, T. P., Parks, J. S., and Rudel, L. L. (1992) *Arterioscler. Thromb.* **12,** 1275–1283
13. Huff, M. W., Telford, D. E., Barrett, P. H. R., Billheimer, J. T., and Gillies, P. J. (1994) *Arterioscler. Thromb.* **14,** 1498–1508
14. Carr, T. P., Hamilton, R. L. J., and Rudel, L. L. (1995) *J. Lipid Res.* **36,** 25–36
15. Field, F. J., Kam, N. T., and Mathur, S. N. (1990) *Gastroenterology* **98,** 539–551
16. Veldhuis, J. D., Strauss, J. F. d., Silavin, S. L., and Kolp, L. A. (1985) *Endocrinology* **116,** 25–30
17. Civen, M., Leeb, J., and Morin, R. J. (1982) *J. Steroid Biochem.* **16,** 817–822
18. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1987) *Current Protocols in Molecular Biology,* **Vols. 1** and 2, John Wiley & Sons, New York
19. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1987) *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Lab., Cold Spring Harbor, NY
20. Marck, C. (1988) *Nucleic Acids Res.* **16,** 1829–1836
21. Devereux, J., Haeberli, P., and Smithies, O. (1984) *Nucleic Acids Res.* **12,** 387–395
22. Bairoch A., Bucher P., and Hofmann, K. (1997) *Nucleic Acids Res.* **25,** 217–221
23. Nevill-Manning, C. G., Wu, T. D., and Brutlag, D. L. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 5865–5871
24. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* **215,** 403–410
25. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) *Nucleic Acids Res.* **25,** 3389–3402
26. Don, R. H., Cox, P. T., Wainwright, B. J., Baker, K., and Mattick, J. S. (1991) *Nucleic Acids Res.* **19,** 4008
27. Yang, H., Cromley, D., Wang, H., Billheimer, J. T., and Sturley, S. L. (1997) *J. Biol. Chem.* **272,** 3980–3985
28. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) *J. Bacteriol.* **153,** 163–168
29. Kozak, M. (1989) *J. Cell Biol.* **108,** 229–241
30. Chang, C. C., Noll, W. W., Nutile-McMenemy, N., Lindsay, E. A., Baldini, A., Chang, W., and Chang, T. Y. (1994) *Somatic Cell Mol. Genet.* **20,** 71–74
31. Matsuda, H., Hakamata, H., Miyazaki, A., Sakai, M., Chang, C. C., Chang, T. Y., Kobori, S., Shichiri, M., and Horiuchi, S. (1996) *Biochim. Biophy. Acta* **1301,** 76–84
32. Tavani, D. M., Nes, W. R., and Billheimer, J. T. (1982) *J. Lipid Res.* **23,** 774–781
33. Cao, G., Goldstein, J. L., and Brown, M. S. (1996) *J. Biol. Chem.* **271,** 14642–14648
34. Botham, K. M. (1992) *Biochem. Soc. Trans.* **20,** 454–459
35. Corton, J. M., and Hardie, D. G. (1992) *Eur. J. Biochem.* **204,** 203–208
36. Billheimer, J. T., Cromley, D. A., and Kempner, E. S. (1990) *J. Biol. Chem.* **265,** 8632–8635
37. Erickson, S. K., Lear, S. R., and McCreery, M. J. (1994) *J. Lipid Res.* **35,** 763–769
38. Guo, Z., Yang, H., and Sturley, S. L. (1996) *Circulation* **94,** I-35
39. Rost, B. (1996) *Methods Enzymol.* **266,** 525–539
40. Doolittle, G. M., and Chang, T. Y. (1982) *Biochemistry* **21,** 674–679
41. Goodman, D. S., Deykin, D., and Shiratori, T. (1964) *J. Biol. Chem.* **239,** 1335–1344
42. Schoonderwoerd, K., Broekhoven-Schokker, S., Hulsmann, W. C., and Stam, H. (1990) *Biochem. J.* **268,** 487–492
43. Einarsson, K., Benthin, L., Ewerth, S., Hellers, G., Stahlberg, D., and Angelin, B. (1989) *J. Lipid Res.* **30,** 739–746
44. Billheimer, J. T., and Gillies, P. J. (1992) in *Advances in Cholesterol Research* (Esfahani, M., and Swaney, J. B., eds) Telford Press, Philadelphia
45. Wilson, J. E. (1995) *Rev. Physiol. Biochem. Pharmacol.* **126,** 65–198